Selective sensitiveness of mesenchymal stem cells to shock waves leads to anticancer effect in human cancer cell co-cultures

Selective Sensitiveness of Mesenchymal Stem Cells to Shock Waves Leads to Anticancer Effect in Human Cancer Cell Co-Cultures

Federica Foglietta[a#], Serena Duchi[b#], Roberto Canaparo[a]*, Greta Varchi[c], Enrico Lucarelli[b], Barbara Dozza[b,d], Loredana Serpe[a]

[a]Department of Drug Science and Technology, University of Torino, Italy

[b]Osteoarticular Regeneration Laboratory, Rizzoli Orthopaedic Institute, Bologna, Italy

[c]National Research Council, Institute for the Organic Synthesis and Photoreactivity, Bologna, Italy

[d]Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, Italy

[#] These authors contributed equally to this work

*** Corresponding author:**

Roberto Canaparo, PhD

Dipartimento di Scienza e Tecnologia del Farmaco, University of Torino

Via Pietro Giuria 13, 10125 Torino, Italy

E-mail: roberto.canaparo@unito.it

Phone: +390116706237 Fax: +390112366237

Abstract

Aim: Mesenchymal stem cells (MSC) possess the distinctive feature of homing in on and engrafting into the tumor stroma making their therapeutic applications in cancer treatment very promising. Research into new effectors and external stimuli, which can selectively trigger the release of cytotoxic species from MSC toward the cancer cells, significantly raises their potential.

Main methods: Shock waves (SW) have recently gained recognition for their ability to induce specific biological effects, such as the local generation of cytotoxic reactive oxygen species (ROS) in a non-invasive and tunable manner. We thus investigate whether MSC are able to generate ROS and, in turn, affect cancer cell growth when in co-colture with human glioblastoma (U87) or osteorsarcoma (U2OS) cells and exposed to SW.

Key findings: MSC were found to be the cell line that was most sensitive to SW treatment as shown by SW-induced ROS production and cytotoxicity. Notably, U87 and U2OS cancer cell growth was unaffected by SW exposure. However, significant decreases in cancer cell growth, 1.8 fold for U87 and 2.3 fold for U2OS, were observed 24 h after the SW treatment of MSC co-cultures with cancer cells. The ROS production induced in MSC by SW exposure was then responsible for lipid peroxidation and cell death in U87 and U2OS cells co-cultured with MSC.

Significance: This experiment highlights the unique ability of MSC to generate ROS upon SW treatment and induce the cell death of co-cultured cancer cells. SW might therefore be proposed as an innovative tool for MSC-mediated cancer treatment.

Keywords

Mesenchymal stem cells; ultrasound; reactive oxygen species; lipid peroxidation; cancer.

1. Introduction

Cancer remains one of the leading causes of morbidity and mortality throughout the world. Several types of cancer do not respond to first line therapies, such as chemotherapy and radiotherapy, and one of the major challenges is to find effective anticancer treatments able to completely eradicate the tumor, while keeping systemic toxicity at minimum [1]. In recent decades, various tumor-targeted therapeutic approaches have been developed at a molecular, cellular and tissue level [2]. In the last years, mesenchymal stem cells (MSC) have received much attention not only as candidates for regenerative medicine regimens, but also for their potential as "smart" delivery systems of chemotherapeutics and nanoparticles [3-8]. MSC are multipotent cells characterized by tropism toward primary and metastatic tumor locations [9,4,8]. It has been shown that specific tumor-derived growth factors secreted by cancer cells, stimulate MSC tropism, including cell-cell and paracrine interactions, thus creating a tight correlation between the two cellular populations [10]. This ability to migrate specifically to tumors has meant that MSC have been used to deliver anticancer and biological agents to tumor tissues [11,12].

Cytotoxic agent-loaded nanoparticles (NPs) have been for instance incorporated into MSC or anchored on their cell surface [13] making NPs engineered MSC act as "Trojan horses" by delivering the therapeutics to the targeted sites. In principle, strategies that exploit MSC as delivery vehicles of cytotoxic compounds, without the need for sophisticated cells engineering would be highly beneficial. In this regard, several recent studies have focused on the development of new anticancer approaches in which the cytotoxicity of conventional drugs or specifically engineered nanoparticles and molecules is triggered by external stimuli, such as light and ultrasound [14-16]. For instance, the activation of responsive molecules by light results in an energy transfer cascade that ultimately leads to the formation of cytotoxic reactive oxygen species (ROS), which are the effectors of apoptotic and necrotic cell death [17]. However, the low tissue penetration depth of light is a major shortcoming of this technique [17]. Therefore, novel systems capable of penetrating

more deeply into tissue and to induce the release of ROS into the tumor stroma are required. In this respect, we have recently introduced a novel non-invasive treatment, which induces tumor cell death by triggering the cytotoxicity of responsive molecules via shock waves (SW) [18,19].

SW have been used for many years in extracorporeal shock wave lithotripsy, while more recent SW applications include drug delivery and gene therapy [20-22]. SW are pulsed ultrasound which are characterized by microsecond pressure surges and sudden positive pressure peak (up to values of about 100 MPa), followed by smaller negative pressure peak (about 10 MPa) [22]. SW can cause direct physical and indirect biological effects that can be amplified by sensitizer molecules [19]. Little is known about the mechanism responsible for the biological effects triggered by ultrasound, though the "intra-membrane cavitation model" reported by Krasovitski et al. [23] is an important attempt to explain the effect. According to this model, ultrasound with specific characteristics can induce modifications in the lipid bilayer of cell membrane leading to intracellular deformations. Moreover, under appropriate conditions, a significant oxidative stress may occur during SW exposure due to the imbalance between ROS production and antioxidant defence [24], resulting in ROS-induced cytotoxicity [25]. However, the *in vivo* treatment of solid tumors by SW alone has been shown to be ineffective [26].

It is currently believed that only unregulated levels of ROS are harmful for cells, while regulated ROS production promotes essential signalling pathways, which control cell functions such as cells proliferation, differentiation and apoptosis. MSC osteogenic and adipogenic differentiation are regulated by the intracellular levels of ROS [27]. Understanding the impact of ROS on MSC may reveal how these cells can be harnessed for therapeutic purposes. Our hypothesis is that upon selective exposure to external SW, MSC could generate a significant amount of ROS able to kill cancer cells, thus functioning as cell-based "ROS-producers" for targeted tumor treatment. By using *in vitro* MSC co-cultures with human glioblastoma (U87) and osteosarcoma (U2OS) cancer cell lines, we demonstrated for the first time that MSC are able to kill cancer cells under the exclusive control of SW irradiation.

2. Materials and Methods

2.1. *Cell lines and culturing*

Bone marrow (BM) samples were obtained from patients who underwent surgery at Rizzoli Orthopaedic Institute (Bologna, Italy); informed consent was given according to a protocol approved by the Local Ethics Committee (Prot.gen. 0004377). Isolation and culture expansion of human MSC was performed as previously described [28]. Briefly, nucleated cells were isolated using a density gradient solution (Ficoll-Paque PREMIUM, Ge Healthcare, Uppsala, Sweden) and plated in culture flasks with α-Modified Minimum Essential Medium (α-MEM, Lonza, Verviers, Belgium), which was supplemented with 20% fetal bovine serum (FBS, Lonza) and 1% GlutaMAX™ (Invitrogen-Life Technologies, Paisley, UK). The cells were cultured in a humidified atmosphere of 5% CO_2 air at 37°C and the medium changed every 3-4 days. When adherent cells reached approximately 70-80% confluence, they were detached by mild trypsinization (TrypLE Select; Gibco Invitrogen Corp., Grand Island, NY, USA) and seeded into new culture flasks at a density of 4×10^3 cells/cm^2 for continued passages. For the experiments, MSC were used between passages 3 and 6, and tumor cells between passages 4 and 25. Human glioblastoma-astrocytoma U87MG-RFP cells (U87-RFP) were kindly provided by Dr Laura Falchetti (CNR, Roma, Italy). Human osteosarcoma U2OS-TUBA1B were purchased from Sigma Aldrich (St. Louis, MO, USA) and the genomic TUBA1B gene was endogenously tagged with a Red Fluorescent Protein (RFP) gene using the CompoZr® Zinc Finger Nuclease technology (U2OS-RFP). The human dermal fibroblast cell line, HDF 106, was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK).

U87-RFP cells were cultured in Minimum Essential Medium Eagle (EMEM, Sigma Aldrich, Milano, Italy), U2OS-RFP cells were cultured in McCoy's 5A (Sigma Aldrich) and HDF 106 cells were cultured in DMEM (Sigma Aldrich). All media were supplemented with 10% FBS (Lonza), 2

mM L-glutamine, 100 UI/mL penicillin and 100 μg/mL streptomycin (Sigma Aldrich). All cell lines were maintained in a humidified atmosphere of 5% CO_2 air at 37°C.

2.2. Intracellular glutathione determination

The intracellular content of glutathione (GSH) of MSC, HDF 106, U87-RFP and U2OS-RFP cells was determined using the Glutathione Assay Kit (Sigma Aldrich), according to manufacturer's instructions. The GSH content (nmol GSH) was normalized to μg of protein for each sample by quantifying cell protein concentration (μg/mL) using the Quant-iT Protein Assay Kit on the fluorometer Qubit (Invitrogen-Life Technologies).

2.3. In vitro SW treatment

For SW experiments, single cell lines (MSC, HDF 106, U87-RFP and U2OS-RFP) and co-cultures (MSC with U2OS-RFP/U87-RFP cells and HDF 106 with U2OS-RFP/U87-RFP cells, at a ratio of 1:3) in the exponential growth phase, were washed three times with phosphate buffered saline (PBS) and harvested by trypsinization. 4.0×10^5 cells were then seeded in PBS into a polystyrene culture dish (Trasadingen, Switzerland) for SW exposure using a piezoelectric device (Piezoson 100; Richard Wolf, Knittlingen, Germany) which generated focused SW at an energy flux density (i.e. energy at the focal point) of 0.22 mJ/mm^2, characterized by a positive pressure peak of 31 MPa, for 1000 impulses at a frequency of 4 impulses/s. SW exposure conditions were chosen, according to the literature, in order to obtain intramembrane cavitation without direct mechanical cell damage [18,29,26,30]. Experiments were carried out in a single SW treatment. Specifically, the focal area, defined as the area in which 50% of the maximum energy is achieved, is assumed to be an elliptical focus cigar with a length of 10 mm in the direction of the shock wave propagation axis and a diameter of 2.5 mm perpendicular to this axis. To control the SW penetration

depth we used an acoustically adapted gel pad of 4.0 cm thickness, which allowed a 5 mm SW penetration depth. The polystyrene culture dish was placed in close contact with the gel pad secured on the transducer using common ultrasound gel.

The effect of SW treatment on single cell lines (MSC, HDF 106, U87-RFP and U2OS-RFP) and on co-cultures (MSC with U2OS-RFP/U87-RFP cells and HDF 106 with U2OS-RFP/U87-RFP) was monitored by fluorescence microscopy (DMI4000B Leica, Wetzlar, Germany), and cells were manually counted 24, 48 and 72 h after SW treatment.

2.4. Cell growth assay

Each experiment was carried out by exposing 4.0 x 10^5 cells to SW and by subsequently seeding 2.0 x 10^4 cells of either single cell line or co-cultured cell sample in 2.0 mL of culture medium in replicates in 6-well culture plates. Thanks to the RFP fluorescent protein present in the two tumor cell lines, it was possible to discriminate between MSC and tumor cells and to obtain the cell count for each cell lines. A total of 5 fields were analysed at a magnification of 10x for each treatment condition in three independent experiments at 24, 48 and 72 h after SW treatment.

Moreover, co-culture cell growth after SW treatment was determined also in the presence of a ROS scavenger in order to investigate the influence ROS production has on cell growth after SW exposure. Briefly, MSC were exposed to the ROS scavenger N-Acetyl-L-cysteine (NAC, Sigma Aldrich), whose primary function is the inhibition of ROS induced cellular damage [31]. MSC were pre-incubated with NAC (5 mM) in culture medium without FBS at 37°C for 1 h, washed in PBS and co-cultured with either U87-RFP or U2OS-RFP for the SW treatment; cell growth was then monitored as previously described. Furthermore, it was decided to investigate whether factors other than ROS were responsible for cancer cell growth decrease. Therefore, we collected MSC culture media at 0.5, 5 and 20 h after the SW treatment of MSC, with the final aim to incubate directly with these media either U87-RFP or U2OS-RFP cells previously plated alone.

2.5. Flow cytometric analyses

ROS generation, cell death and lipid peroxidation were assessed using flow cytometric assays on a C6 flow cytometer (Accurri Cytometers, Milano, Italy). In order to evaluate whether SW exposure induces ROS production in different cell lines, 2,7-dichlorofluorescein (DCF) diacetate (DA) (DCF-DA; Molecular Probes, Carlsbad, CA, USA) was used as an intracellular probe for oxidative stress detection. DCF-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolysed, by intracellular esterases, to the non-fluorescent DCFH. This is in turn rapidly oxidized in the presence of peroxides to highly fluorescent DCF upon oxidation by ROS [32]. Briefly, cells were incubated with 10 μM DCF-DA for 30 min, washed with PBS, trypsinized, subjected to SW exposure and analysed at 5, 15, 30 and 60 min. ROS production was expressed as integrated median fluorescence intensity (iMFI), which is the product of ROS-producing cell frequency and the median fluorescence intensity of the cells [33]. ROS production by MSC and HDF 106 after SW exposure was investigated also in the NAC (Sigma Aldrich) pre-treated cells. Briefly, MSC were pre-incubated with NAC (5 mM) in culture medium without FBS at 37°C for 1 h. Cells were then PBS washed and incubated with DCF-DA, as previously described.

Cell death was evaluated using the Dead Cell Apoptosis Kit with Annexin V-Alexa Fluor® and propidium iodide (PI, Life Technologies, Milano, Italy). 4.0×10^5 cells were treated and cell death was evaluated 24 h after SW treatment. Briefly, cells were detached with trypsin and washed with PBS at 1,500 rpm for 5 min and then re-suspended with 1x Annexin-binding buffer and stained with Annexin V-Alexa Fluor® and PI. Sample analyses were carried out at 488 nm excitation to measure Annexin V-Alexa Fluor® and at 530 nm to measure PI, respectively. 10,000 events were considered in the analyses and any cell debris that displayed low forward light scatter and side light scatter was excluded from the analyses. The two different staining types allowed us to identify apoptotic (Annexin V-Alexa Fluor® positive) and necrotic cells (Annexin V-Alexa Fluor® and PI

positive) and tell them apart from viable cells (Annexin V-Alexa Fluor® and PI negative). All analyses were performed using FCS Express software version 4 (BD Bioscience, Milano, Italy).

The Image-iT® Lipid Peroxidation Kit (Life Technologies) was used, according to manufacturer's instructions, to investigate whether SW treatment was able to induce damage via the oxidative degradation of cellular lipids. The BODIPY 581/591 C11 reagent is a fluorescent lipid peroxidation reporter molecule that shifts its fluorescence from red to green when challenged with oxidizing agents [34]. Briefly, 4.0×10^5 cells were treated with SW at an energy flux density of 0.22 mJ/mm^2 for 1000 impulses (4 impulses/s). The reagent was added to cells for 30 min incubation at 37°C and then lipid peroxidation was evaluated 12 and 24 h after SW treatment. Cells were also treated with cumene hydroperoxide (200 mM) for 2 h without SW exposure as a positive control. Cells were detached by trypsin, washed with PBS and data were acquired by reading fluorescence at two separate wavelengths: one at excitation/emission of 581/591 nm for the reduced dye, and the other at excitation/emission of 488/510 nm for the oxidized dye. The ratio between emission fluorescence intensities at 590 nm to 510 nm gave us the read-out for lipid peroxidation in cells.

2.6. Statistical analyses

Data are shown as average values ± standard deviation of three independent experiments. Statistical analyses were performed on Graph-Pad Prism 6.0 software (La Jolla, CA, USA); two-way analysis of variance and Bonferroni's test were used to calculate the threshold of significance. The statistical significance threshold was set at $p < 0.05$.

3. Results

3.1. Intracellular glutathione level

GSH is an important intracellular low molecular weight tripeptide thiol that plays numerous important biological functions, including protecting cells from toxic compounds such as ROS [35]. Therefore, we determined the GSH level in all the considered cell lines. No significant differences of intracellular GSH levels were observed in MSC, HDF 106 and U87-RFP cells, whereas a significantly higher GSH level was observed in U2OS-RFP cells (Fig. 1).

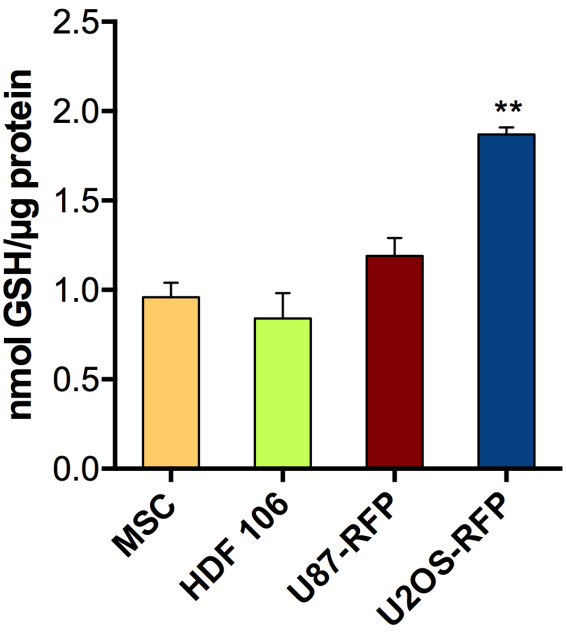

Fig. 1. Intracellular GSH content according to cell type. The reduced GSH content of human MSC, HDF 106, U87-RFP and U2OS-RFP cells was measured at a basal level, i.e. in untreated cells, and was expressed as nmol/µg protein. Statistical significance between MSC and the other cell lines: ** $p < 0.01$.

3.2. Effect of SW treatment on cell growth and ROS production in single cell lines

MSC, HDF 106, U87-RFP and U2OS-RFP cell lines were separately exposed to the same SW treatment. As MSC and HDF 106 cells displayed a cell population doubling time that was roughly 2 times lower than that of U87-RFP and U20S-RFP cells, cell growth data are expressed as a percentage of control cells, i.e. untreated cells at the corresponding time point. A significant decrease in cell growth was observed only in MSC from 24 to 72 h (Fig. 2A, 80.31 ± 7.50% at 24 h, 78.00 ± 8.17% at 48 h and 64.43 ± 14.96% at 72 h) and in HDF 106 at 24 h (Fig. 2B, 79.75 ± 1.77% at 24 h), whereas U87-RFP and U2OS-RFP cell growth was unaffected by SW exposure (Fig. 2C, D). We then carried out the DCF-DA cytofluorimetric assay in order to quantitatively measure the ROS production that was induced by SW exposure in MSC, HDF 106, U87-RFP and U2OS-RFP. As compared to untreated MSC, SW-treated MSC showed a statistically significant ROS production from 1 up to 60 min after SW treatment (Fig. 3A, $p < 0.001$). Moreover, the ROS production after SW exposure of NAC pre-treated MSC was strongly decreased indicating a specificity of ROS production induced by SW treatment (Fig. 3B). A lower but statistically significant ROS production was observed also in HDF 106 cells from 15 up to 30 min after SW treatment, as compared to untreated HDF 106 cells (Fig. 3C, $p < 0.05$). Interestingly, no significant increase in ROS production was observed in either U87-RFP or U2OS-RFP cells after SW treatment, as compared to untreated cancer cells (Fig. 3D-E). Therefore, it is possible to state that MSC are more sensitive to SW treatment than the other cell lines, according to the data of intracellular GSH level (Fig. 1), cell growth (Fig. 2) and ROS production (Fig. 3).

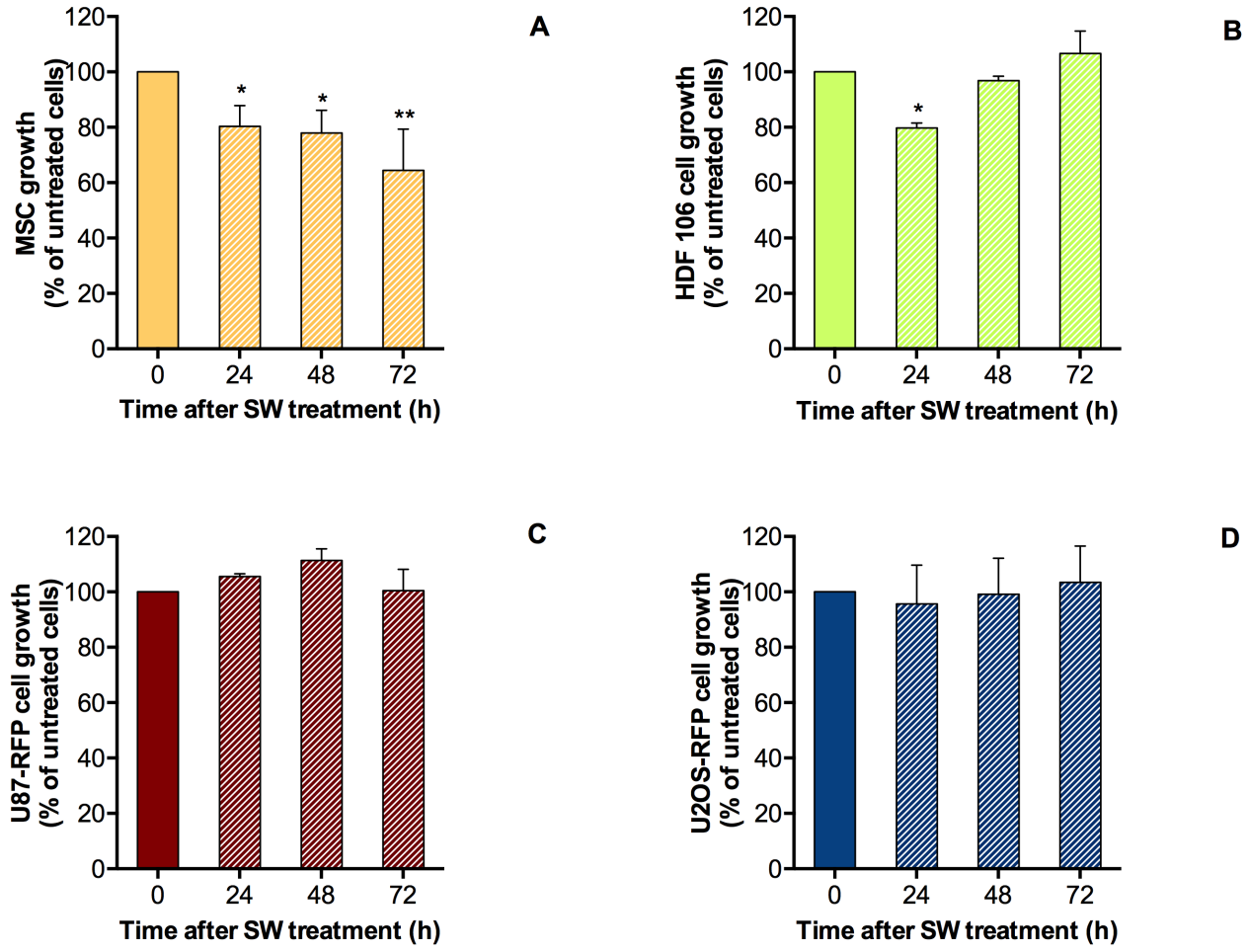

Fig. 2. Effects of SW treatment on the single cell line growth. MSC (A), HDF 106 (B), U87-RFP (C) and U2OS-RFP (D) cells were exposed to SW treatment at an energy flux density (EFD) of 0.22 mJ/mm^2 for 1000 impulses (4 impulses/s). Cell growth was evaluated after 24, 48 and 72 h by fluorescence microscopy. Statistical significance between untreated cells (full bars) and SW-treated cells (dashed bars) at the respective time point: * $p < 0.05$, ** $p < 0.01$.

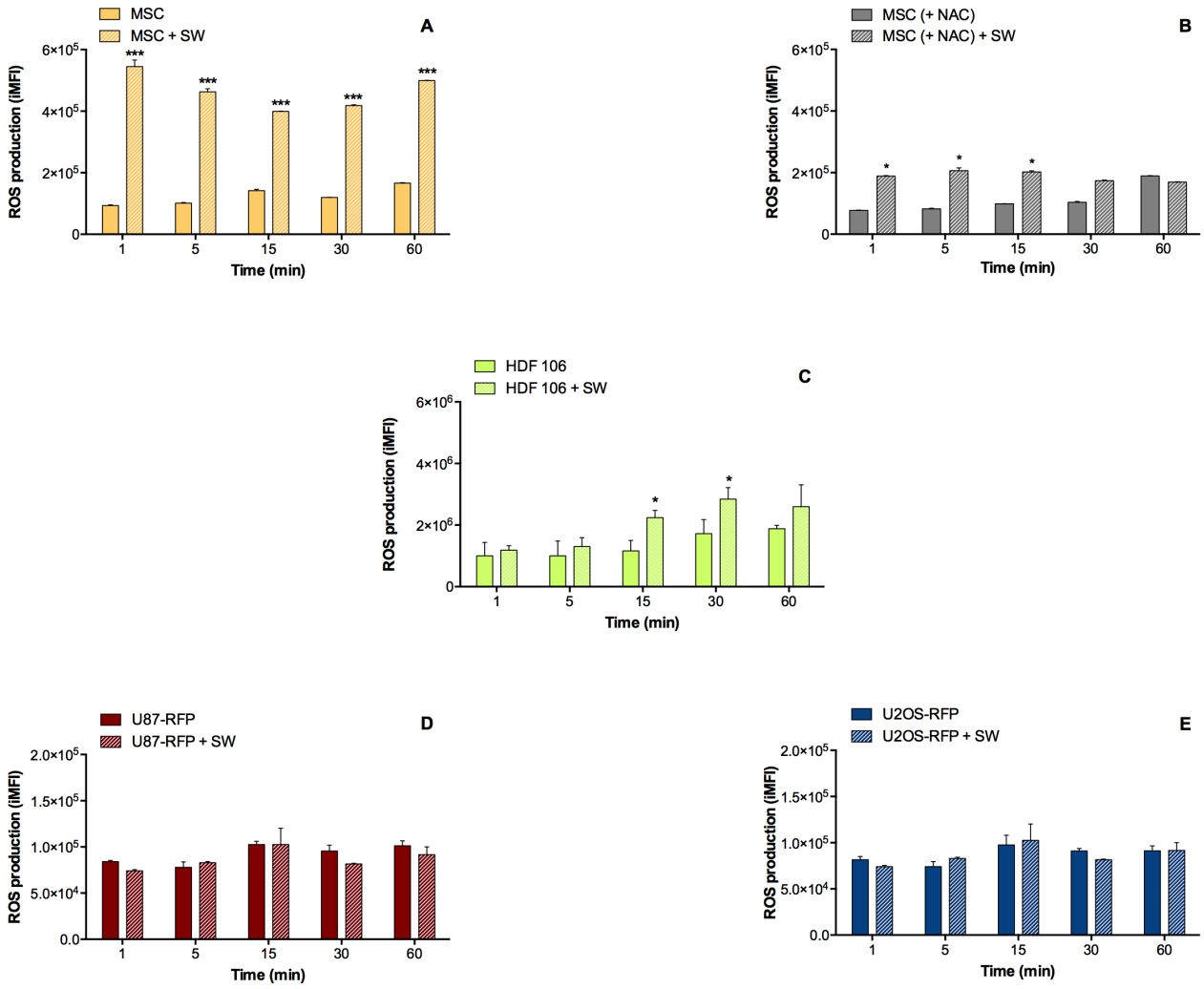

Fig. 3. ROS production of single cell lines after SW treatment. MSC (A, B), HDF 106 (C), U87-RFP (D) and U2OS-RFP (E) cells were exposed to SW treatment at an energy flux density (EFD) of 0.22 mJ/mm^2 for 1000 impulses (4 impulses/s) and ROS production at 1, 5, 15, 30 and 60 min was investigated using the DCF-DA assay. The ROS production of MSC cells was also determined by incubating cells with the ROS scavenger N-Acetyl-L-cysteine (NAC, 5 mM; panel B) 1 h before SW treatment (EFD 0.22 mJ/mm^2 for 1000 impulses, 4 impulses/s). Statistical significance between untreated cells or co-cultures (full bars) and SW-treated cells or co-cultures (dashed bars): * $p < 0.05$, *** $p < 0.001$.

3.3. Effect of SW treatment on cell growth and ROS production of co-cultures

Fig. 4 reports the effect of a single SW treatment on MSC that were co-cultured with either U87-RFP or U2OS-RFP cells. A statistically significant decrease in cell growth was observed in both co-cultures (Fig. 4A-B). Specifically, 24 h after SW treatment we observed a cancer cell growth decrease of 1.8 fold in the MSC/U87-RFP co-culture (Fig. 4A, 41.32 ± 10.25% at 24 h) and of 2.3 fold in the MSC/U2OS-RFP co-culture (Fig. 4B, 32.80 ± 3.78% at 24 h), both results are compared to the cancer cell growth of the respective untreated co-cultures (Fig. 4A and B, $p < 0.001$). Moreover, a less significant cancer cell growth decrease was observed 48 and 72 h after SW treatment in both cancer cell co-cultures (Fig 4A, 54.63 ± 3.40% at 48 h, 58.68 ± 4.35% at 72 h and Fig. 4B, 59.95 ± 6.53% at 48 h, 56.23 ± 3.12% at 72 h, respectively) as compared to the cancer cell growth of the respective untreated co-cultures (Fig. 4A and B, $p < 0.01$).

Furthermore, it was investigated whether the SW-induced tumor cell number decrease could be ascribed to the ROS produced by MSC upon SW treatment. Therefore, we performed the same experiments, but with MSC pre-treated with 5 mM NAC before SW treatment. As reported in Fig. 4C and D, a lower decrease in cancer cell number was detected in the co-cultures with NAC pre-treated MSC; specifically, we did not observe the same significant reduction in the cell growth in U87-RFP cells at 24 h (Fig. 4C, $p < 0.01$) and in U2OS-RFP cells at 24 h (Fig. 4D, $p < 0.05$), as compared to the respective SW treated co-cultures in which MSC were not exposed to NAC (Fig. 4A and B). Moreover, no cancer cell number decrease was observed for MSC incubated with NAC and co-cultured with either U87-RFP or U2OS-RFP 48 and 72 h after SW treatment (Fig. 4C and D).

The effect of SW treatment on co-cultures of the same cancer cells, i.e. U87-RFP and U2OS-RFP, with HDF 106 cells was investigated in order to define whether MSC are characteristic/selective in inducing a SW-mediated cell growth decrease of co-cultured cancer cells.

SW treatment did not affect cancer cell growth (Fig. 4E-F) when HDF 106 cells were co-cultured with either U87-RFP or U2OS-RFP cells.

Furthermore, MSC alone were exposed to the same SW treatment and their collected culture media were incubated with either U87-RFP or U2OS-RFP cells previously cultured alone. This was done in order to investigate whether further factors, other than ROS formed upon SW exposure, might be responsible for the observed cancer cell growth decrease. Indeed, no change in cancer cell growth was observed up to 72 h (data not shown), suggesting that ROS generated in situ by MSC under SW exposure, are the only effectors of cancer cell growth decrease.

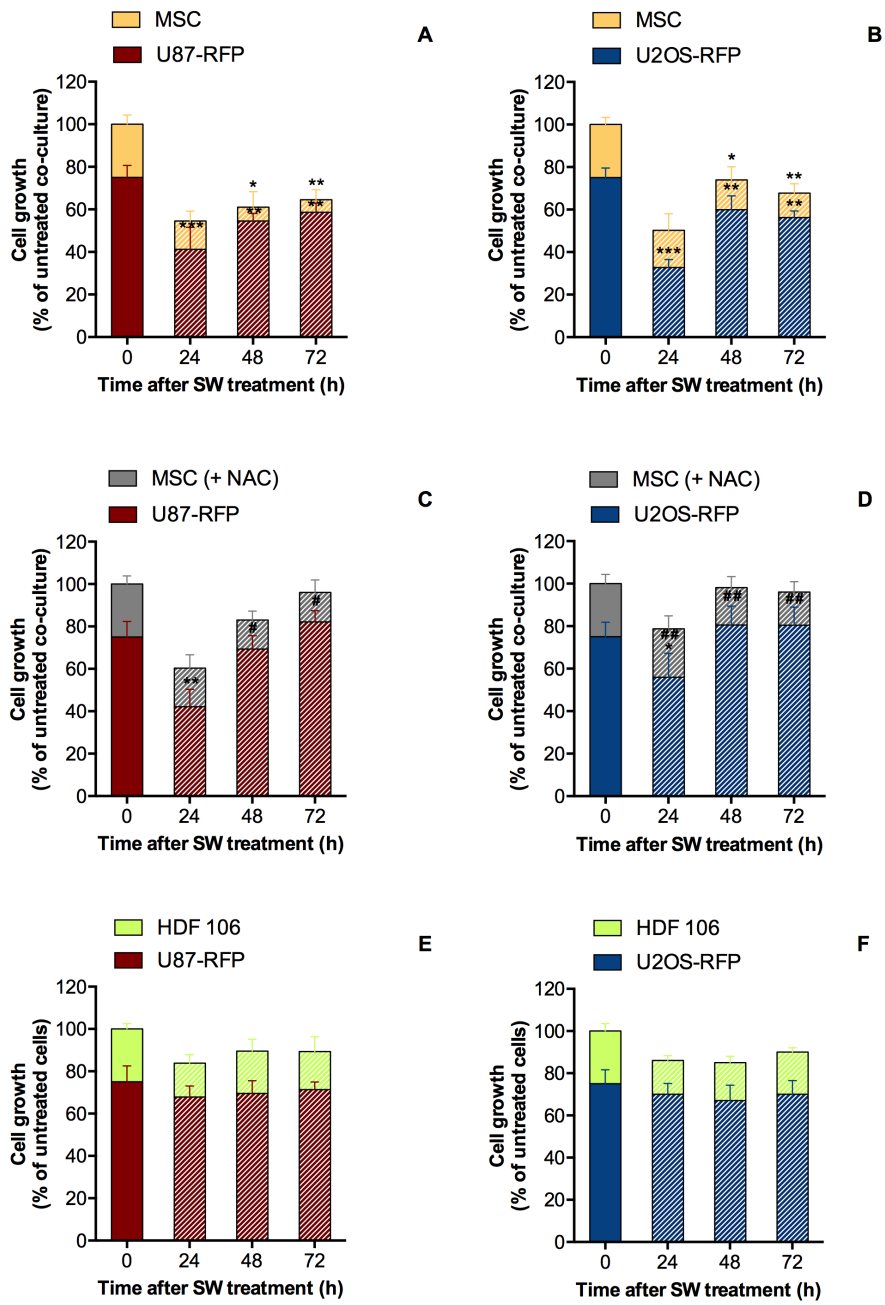

Fig. 4. Effect of SW treatment on the co-culture cell growth. Co-cultures of MSC and either U87RFP (A) or U2OS-RFP (B) cells were exposed to SW treatment at an energy flux density (EFD) of 0.22 mJ/mm^2 for 1000 impulses (4 impulses/s). The same SW treatment was carried out also on co-cultures of MSC, previously incubated with the ROS scavenger N-Acetyl-L-cysteine (NAC, 5 mM) for 1 h, with either U87-RFP (C) or U2OS-RFP (D) cells. The same SW treatment was carried out also on co-cultures of HDF 106 with either U87-RFP (E) or U2OS-RFP (F) cells as a co-culture control. Cell growth was evaluated at 24, 48 and 72 h by fluorescence microscopy.

Statistical significance between untreated co-cultures (full bars) and SW-treated co-cultures (dashed bars) at the respective time point: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and between co-cultures with MSC or with MSC incubated with NAC (+ NAC): $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$.

3.4. Lipid peroxidation on MSC co-cultures after SW treatment

The analysis of lipid peroxidation was performed in order to investigate whether ROS production by MSC under SW exposure was able to induce downstream events in the co-cultures that undergo cancer cell growth decrease. As reported in Fig. 5, 12 h after SW treatment, a large amount of reduced lipid intermediates were detected in all the considered cell models (high reduced 590/oxidized 510 ratio). Twenty-four hours after SW exposure, a lower ratio of reduced 590/oxidized 510 was observed, accounting for the presence of a large amount of oxidized lipid intermediates (Fig. 5). In particular, we observed that both MSC alone and in co-culture with U2OS-RFP cells became positive to the green channel after SW treatment, indicating the presence of oxidized lipid peroxidation intermediates. Specifically, by comparing the reduced 590/oxidized 510 ratio 12 h and 24 h after SW treatment, a significant formation of oxidized lipid intermediates could be detected; in particular a nearly 2.5, 1.8 and 4.4 fold increase was observed for MSC ($p < 0.01$), MSC/U87-RFP co-culture ($p < 0.01$) and MSC/U2OS-RFP co-culture ($p < 0.001$), respectively (Fig. 5).

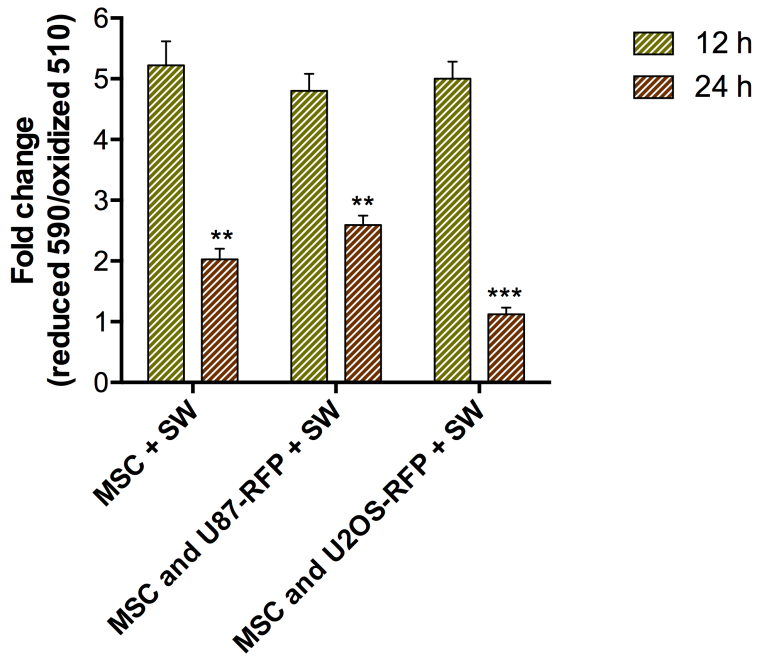

Fig. 5. Lipid peroxidation occurrence after SW treatment. MSC and MSC co-cultured with either U87-RFP or U2OS-RFP cells were exposed to SW treatment at an energy flux density (EFD) of 0.22 mJ/mm^2 for 1000 impulses (4 impulses/s). Lipid peroxidation was evaluated 12 and 24 h after SW treatment by cytofluorimetric assay. Statistical significance between lipid peroxidation 12 h and 24 h after SW treatment: ** $p < 0.01$, *** $p < 0.001$.

3.5. Cell death analysis on MSC co-cultures after SW treatment

A cell death analysis by flow cytometric assay was performed 24 h after SW treatment to determine whether the cancer cell growth decrease induced by SW treatment of MSC co-culture was due to apoptosis and/or necrosis. After SW treatment, no significant increase in apoptotic or necrotic cells was observed in both cancer cell lines compared to untreated cells, whereas a significant increase in necrotic cells was observed in MSC (Tab.1, $p < 0.05$), as compared to untreated cells. Interestingly, a significant increase in both apoptotic and necrotic cells was observed, as compared to untreated co-cultures, in the MSC co-cultures with U87-RFP cells (Tab.1, $p < 0.01$) and U2OS-RFP cells (Tab.1, $p < 0.01$) after SW treatment.

Tab.1. Cell death detection 24 h after SW treatment.

	Live cells	**Apoptotic cells**	**Necrotic cells**
MSC	86.84 ± 10.52	7.10 ± 1.13	6.06 ± 8.67
MSC + SW	75.35 ± 6.01	9.55 ± 3.61	15.10 ± 3.75 *
U87-RFP	92.23 ± 8.17	2.15 ± 0.78	5.62 ± 0.95
U87-RFP + SW	87.76 ± 5.73	5.51 ± 2.30	6.73 ± 1.84
U2OS-RFP	87.39 ± 9.42	5.12 ± 0.64	8.03 ± 1.12
U2OS-RFP + SW	90.42 ± 5.70	5.61 ± 3.23	4.23 ± 1.20
MSC and U87-RFP	92.60 ± 4.95	4.80 ± 1.13	2.60 ± 0.57
MSC and U87-RFP + SW	59.08 ± 0.18 **	17.27 ± 4.48 **	23.65 ± 4.22 **
MSC and U2OS-RFP	89.85 ± 2.62	4.00 ± 1.41	6.15 ± 1.77
MSC and U2OS-RFP + SW	58.86 ± 6.72 **	16.67 ± 3.52 **	24.47 ± 3.58 **

Statistical significance between untreated cells or co-cultures and SW-treated cells or co-cultures: * $p < 0.05$, ** $p < 0.01$.

4. Discussion

The last decade has witnessed the publication of a large number of studies that describe MSC as effective anticancer tools thanks to their selective ability to migrate towards tumor cells and deliver cytotoxic drugs, i.e. the “Trojan horse” concept. Indeed, tumors can produce a large and continuous amount of cytokines, chemokines and inflammatory mediators, which are signals capable of recruiting respondent cell types, including MSC [36,6,37]. Our recent work on the use of MSC as drug delivery vehicles for anti-tumor therapeutic applications, has prompted us to find new approaches/techniques that can induce the release of cytotoxic ROS from MSC toward tumor cells [3].

SW have recently gained recognition as a valuable means for the non-invasive and tunable induction of specific biological effects [19,20,38]. It is generally accepted that one of the main effectors of the non-thermal interactions between ultrasound and biological tissues is acoustic cavitation and that it is able to generate short-lived species, such as ROS and free radicals, thanks to its high-energy release [39,40]. It has been reported that ultrasound alone, which results in a cavitation process, can induce time-dependent apoptotic cell death in *in vitro* liquid tumors, such as human myeloid leukemia and histiocytic lymphoma [41,42]. Indeed, intramembrane cavitation may be the mechanism underpinning ultrasound-induced intracellular ROS production [23].

The purpose of this study is to evaluate whether SW exposed MSC are able to impact on cancer cell growth, via MSC/SW mediated ROS generation, in *in vitro* co-culture models. In particular, we investigated two different co-culture systems, MSC + human glioblastoma (U87-RFP) and MSC + osteosarcoma (U2OS-RFP). The choice of these tumor cell lines is in line with the urgent need to find efficient therapeutic systems to treat cancers located in poorly accessible areas, such as bones and the brain [43,44].

Our first experiment investigated the amount of intracellular glutathione (GSH), a potent antioxidant that it is generally overexpressed in cancer cells [45], in all the considered cell lines, to

find a correlation between GSH content and sensitivity to ROS action. Our data show that a substantially higher amount of GSH was present in U2OS-RFP cells. These were followed by U87-RFP, HDF 106 and finally MSC, indicating that cancer cells, mainly U2OS-RFP, may display a better defensive response to oxidative stress. We therefore supposed that cell growth would decrease in line with measured GSH levels once cell lines were exposed to the SW treatment. Interestingly, significant decreases in cell growth were only observed only in MSC.

We then evaluated the ability of all the considered cell lines, MSC, HDF 106, U2OS-RFP and U87-RFP, to generate ROS upon SW exposure in order to strengthen our hypothesis of a particular ROS generating mechanism existing between SW and MSC. Strong and significant ROS production was only observed in MSC for up to 60 minutes after SW treatment, highlighting the fact that MSC were the most sensitive to SW exposure of all the treated cell lines. Moreover, pre-incubation of MSC with a ROS scavenger, i.e. NAC, significantly decreased MSC-mediated ROS production, as compared to non-pre-treated cells, emphasising the selectivity of the MSC response to SW exposure. These combined data point to the peculiar sensitivity that MSC possess to SW exposure.

MSC/SW mediated ROS generation was able to induce a significant decrease in cancer cell growth over time and in both cancer cell lines in our co-culture experiments. In particular, the reduction in cancer cell growth was very significant (> 40%) in both co-cultures 24 h after SW exposure, whereas it was less prominent at 48 and 72 h after treatment. Conversely, the pre-incubation of MSC with NAC induced a significantly lower decrease in co-culture cell growth, thus confirming the ROS-mediated action of the MSC/SW system [46,47]. Our results are in agreement with a previously reported study, which describes the generation of ROS during cavitation and how the presence of a free radical-scavenger diminishes cells damage [40].

In order to confirm that the decreased cancer cell growth in SW-treated co-cultures was selectively determined by MSC/SW-induced ROS production, we investigated cancer cell growth on two separate co-cultures of U87-RFP and U2OS-RFP cells with human dermal fibroblast

(HDF106) instead of MSC. Dermal fibroblasts are considered to be mature mesenchymal cells and are particularly abundant in the connective areas of each organ and tissue [48]. Moreover, it has been reported that MSC and fibroblasts share many characteristics in differentiation, proliferation potential, distribution, phenotype and immunoregulation [48,49]. A slight but statistically significant decrease in cell growth was only observed in HDF 106 cells treated alone 24 h after SW exposure. This may be due to the low level of GSH present, but no decrease in HDF 106 and cancer cell growth was observed when HDF106/U87-RFP and U2OS-RFP co-cultures where exposed to SW. Once again this data confirm the peculiar biological response that MSC have upon SW exposure; producing ROS which are able to kill co-cultured cancer cells.

Since MSC ROS production after SW exposure seems to be the mechanism involved in killing cancer cells in co-culture models, it was decided that we evaluate the degree of lipid peroxidation after SW treatment in MSC alone and in co-culture models. Indeed, the lipid peroxidation of polyunsaturated fatty acids is one of the major effects induced by oxidative stress and one that can dramatically alter cell integrity [50]. We were able to observe a significant increase in lipid peroxidation 24 h after SW treatment both in MSC and in MSC/cancer cell co-cultures. The significant reduction in cancer cell growth observed in the co-culture models under SW exposure, with a MSC/cancer cell ratio of 1:3, is most likely due to the ROS produced by MSC, which leads to the significant lipid peroxidation both in MSC and in cancer cells. Our data agree with recent work by Leung and co-workers which reported the role of ultrasound in inducing lipid peroxidation [51].

It has also been reported that ultrasound-induced ROS generation is able to trigger apoptotic changes [52]. Therefore we performed a cytofluorimetric analysis of SW-treatment induced cell death after 24 h, in order to investigate whether cancer cell growth reduction was due to apoptotic and/or necrotic cell death. Our results highlighted very significant increases in both apoptotic and necrotic cells in SW-treated MSC/cancer cell co-cultures, as compared to untreated co-cultures, as

the cell death of MSC and cancer cells was due to the ROS burst selectively induced in MSC by SW exposure.

Overall, our data show a MSC's peculiar sensitiveness to SW exposure. It is well known how the microenvironment may influence the stem cell behavior, however, only a few studies have investigated the role of mechanical stimulations in this contest [53-55]. Our results lead us to take the risk of speculating about how an induced-mechanical stress may provoke different cellular effects according to cell type dependent features, such as the cytoskeleton. This can lead to differences in intracellular cavitation generation under SW exposure, that may not be effective in producing reactive oxygen species in some cells, such as cancer cells, but does so in other cells, such as MSC [56]. It is worth nothing that our experiments were carried out using SW that are characterized by low positive pressure peak (i.e., 31 MPa) that may fit well with the theory, known as "bilayer sonophore", reported by Krasovitski B et al. [23]. This means that the bilayer membrane is capable of transforming the oscillating acoustic pressure waves into nanometric and micrometric intracellular deformations, under appropriate conditions, which are, in turn, able to induce intracellular cavitation without provoking direct mechanical damage to the cell membrane.

5. Conclusion

This study represents the first investigation on the effects of SW treatment on the cell growth of human cancer cells co-cultured with mesenchymal stem cells. We demonstrated that MSC, under SW exposure, work as “ROS-producers” able to induce significant ROS production that in turn triggers a cascade of events in co-cultured cancer cells, including lipid peroxidation that leads to cancer cell death. These encouraging preliminary results could pave the way for investigations into whether, and how, SW can be used for the stem cell therapy of malignancies.

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Conflict of Interest Statement

All the Authors declare that there are no conflicts of interest.

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